

**Role of macrophage secretions on rat polycystic ovary: Its effect on apoptosis.**

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## Abstract

Polycystic ovarian syndrome is the most common endocrine disorders among women of reproductive age. Little is known about its etiology, although the evidence suggests an intrinsic ovarian abnormality in which endocrine, metabolic, neural and immune factors would be involved. In this work, the effects of macrophage (MO) secretion on ovarian apoptosis in a polycystic ovary syndrome rat model (PCO rat) induced by estradiol valerate are studied. Spleen macrophages secretions were used to stimulate ovaries, and ovarian interstitial and granulosa cells, from both PCO and control rats. Ovarian hormones and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) were measured by radioimmunoassay; ovarian mRNA levels of Bax, Bcl2 and NFkB by RT-PCR; and ovarian inducible nitric oxide synthase (iNOS) by western blot. The number of apoptotic cells was evaluated by TUNEL. In PCO ovary, the MO secretions from PCO rats increased the Bax and NFkB mRNA expressions, and increased TUNEL staining in both granulosa and theca cells. In addition, the PCO MO secretions produced a decrease of nitric oxide release, iNOS protein level and PGE<sub>2</sub> content in the PCO ovary, and it also induced an increase of androstenedione production by PCO interstitial cells, in comparison with control MO secretions. Considering these results and knowing that testosterone stimulates tumour necrosis factor- $\alpha$  production by PCO MO, modifying ovarian response by increasing androstenedione, it is reasonable to suggest that the increase of androgens stimulated in ovarian cells by PCO MO secretions could in turn stimulate the cytokine production from MO, thus maintaining an apoptotic vicious cycle in the PCO ovary.

## Introduction

The bidirectional communication between the neuroendocrine and immune system is firmly recognized. A number of hormonal and neuropeptide mediators have been shown to influence

49 immune development and function in both healthy and diseased individuals (Bilbo & Klein, 2012).  
50 The interactions between ovary and immune cells and the products such as steroids, peptide  
51 hormones, prostaglandins, growth factors and cytokines play a pivotal role in the regulation of  
52 ovarian function (Basedovsky & del Rey 1996, Taub 2008).  
53 The macrophages, the most abundant immune cells within the ovary, have been localized in thecal,  
54 luteal and interstitial tissue compartments and in the atretic follicle, in both animal and human. Their  
55 distribution fluctuates throughout the ovarian cycle with the highest numbers being present at  
56 proestrus and diestrus, strongly indicating hormonal regulation (Wu *et al.* 2004, 2007, Turner *et al.*  
57 2011). Macrophages express functional sex hormone receptors and secrete nitric oxide (NO), along  
58 with tumor necrosis factor alpha (TNF $\alpha$ ), prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), interleukins (IL)-1, IL6, IL10,  
59 IL12 and many other cytokines and growth factors that regulate ovarian function (Gallinelli *et al.*  
60 2003, Sirotkin *et al.* 2011). There is evidence that NO decreases ovarian steroidogenesis by  
61 inhibiting the steroid acute regulatory protein, 3 $\beta$ -hydroxysteroid dehydrogenase (3 $\beta$ -HSD) and  
62 cytochrome p450 side chain cleavage gene expression (Rekawiecki *et al.* 2005). Furthermore, TNF $\alpha$   
63 is a multifunctional cytokine that influences the reproductive axis, inducing changes that closely  
64 resemble those found in patients with hyperandrogenism (Thathapudi *et al.* 2014). TNF $\alpha$  plays a  
65 key role in follicular development and atresia, decreases ovarian cell viability and proliferation, and  
66 stimulates apoptosis in cultured ovarian follicles (Greenfeld *et al.* 2007). When TNF $\alpha$  binds to its  
67 receptor (TNFR1), caspase-8 and caspase-3 are cleaved and activated, and the induction of I $\kappa$ B  
68 phosphorylation and degradation activate the nuclear factor  $\kappa$ B (NF $\kappa$ B). Subsequently, NF $\kappa$ B  
69 translocates into the nucleus where it can activate the transcription of certain genes, particularly  
70 those involved in immune and inflammatory responses (Xiao 2002). In addition, PGE<sub>2</sub> modulates a  
71 variety of physiological processes including the production of inflammatory cytokines (Kuroda &

Yamashita 2003). PGE<sub>2</sub> and their receptor subtypes, EP2 and EP4, are involved in the rat ovarian follicle growth (El-Nefiawy *et al.* 2005).

It is known that a fine balance between pro- and anti-apoptotic factors may determine whether a follicle will continue developing or undergo atresia. Regulation of apoptotic signaling in the ovary is achieved by the Fas system and Bcl2 family, including Bcl2 (antiapoptotic) and Bax (proapoptotic) proteins. The increased expression of the Bax death susceptibility gene coincides with the induction of apoptosis in granulosa cells during atresia *in vivo* and *in vitro* (Tilly 2001). Several studies suggest a cross-talk between the Fas/FasL system-induced apoptosis and the nitric oxide-mediated antiapoptotic pathway in ovarian follicle atresia (Chen *et al.* 2005, Krysko *et al.* 2008). Also, it has been shown that androgen and androgen receptor promote Bax-mediated apoptosis in prostate cancer cells (Lin *et al.* 2006). Furthermore, it is known that PGE<sub>2</sub> promotes proliferation and survival of cells through apoptosis inhibition (Sheng *et al.* 1998, Rask *et al.* 2006).

Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder in women of reproductive age, characterized by anovulation, hyperandrogenemia and/or hyperandrogenism, and frequently, insulin resistance and hyperinsulinemia (Goodarzi *et al.* 2011). Ovaries from most women affected by PCOS are characterized by thecal/interstitial hyperplasia and increased expression of steroidogenic genes, leading to greater androgen biosynthesis and cytochrome P450c17 alpha gene expression (Nelson-Degrave *et al.* 2005).

Several studies suggest that the immune regulation may be involved in the etiology of PCOS (Amato *et al.* 2003, Niccoli *et al.* 2011). Furthermore, neuroimmunomodulatory factors such as dopamine, have been associated to this disorder etiology (Gómez *et al.* 2011). We have shown a functional relationship between the ovarian androgens and immune cells in a rat model of PCOS induced by estradiol valerate (EV). The differential steroidogenic ability of macrophage secretions from those rats is associated to the *in vitro* testosterone environment. Testosterone, probably acting

on macrophage androgen receptor, induces a greater release of  $\text{TNF}\alpha$ , modifying ovarian response by increasing androstenedione and slightly decreasing estradiol without affecting progesterone (Figuerola *et al.* 2012).

There is evidence that dysfunction of granulosa cells may contribute to abnormal folliculogenesis, although the underlying mechanism remains to be determined. It has been demonstrated that there are significant differences in the rate of cell death and proliferation in granulosa cells in PCOS patients compared to women with regular ovulatory cycles. This was associated with decreased expression of apoptotic effectors and increased expression of a cell survival factor (Das *et al.* 2008). However, the role of immune cells on the apoptosis of polycystic ovary remains obscure.

It is known that the EV-PCO model is characterized by acyclicity, anovulation, hyperandrogenemia and polycystic ovarian morphology, and is associated with increased sympathetic activity resembling PCO (Brawer *et al.* 1986, Lara *et al.* 1993, Forneris *et al.* 2003), without metabolic disturbances such as dyslipidemia, insulin resistance or obesity (Stener-Victorin *et al.* 2005).

Therefore, the aim of this study is to investigate in a rat model of PCOS induced by EV (PCO rats):

1) whether secretions of macrophages induce apoptosis in the ovary, and 2) if macrophage secretions modify the ovarian  $\text{PGE}_2$  and nitric oxide levels, and consequently the androstenedione response, in order to establish their relationship with the ovarian apoptosis. For that, secretions of macrophages from spleen were used to stimulate isolated ovarian cells and ovaries from PCO rats.

## Materials and Methods

### *Reagents*

Estradiol valerate (EV), fetal bovine serum and RPMI 1640 medium were purchased from Sigma (St. Louis, MO, USA). TRIzol reagent was obtained from Invitrogen/Life Technology. 1,2,6,7- $[\text{}^3\text{H}]$ -

Prostaglandin E (107.0 Ci/mmol) was provided by New England Nuclear Products (Boston, MA, USA). Other reagents and chemicals were of analytical grade.

### ***Animals and treatment***

Adult Holtzman cycling rats showing at least two regular 4-day cycles were used. They were housed in a controlled environment (22–24 °C, 12 h light–12 h dark). Water and food were available *ad libitum*. Animals were handled according to the procedures approved in the UFAW Handbook for the Care and Management of Laboratory Animals - vol 1- Terrestrial vertebrates-edn 7, edited by T Poole (1999), and the experimental protocol was approved by the Committee for the Use and Care of Animals of the National University of San Luis.

Two groups of rats were used. The first group consisted in PCO rats to which the PCOS model was induced at 60 days of age. This was accomplished by the administration of EV as a single intramuscular injection (2 mg/rat diluted in 0.2 ml corn oil) in order to resemble, in some aspects, the human syndrome (Brawer 1986). The second group, non-PCO rats (control rats), was injected with vehicle alone. All experiments were performed two months after EV injection, at which point cystic follicles were observed by light microscopy. Cystic follicles were devoid of oocytes and exhibited a large antral cavity, an enlarged thecal cell layer, and a thin granulose-cell compartment. Since PCO rats predominantly showed cornified vaginal smears, control rats were sacrificed by decapitation on estrus day. The spleen and the ovaries were removed to obtain macrophages (MO) for culture and ovarian incubations, respectively.

### ***Macrophage culture***

The culture of MO was performed as described previously (Figuerola *et al.* 2012). Briefly, the spleens from PCO and control rats were washed in saline solution and pressed through a sterile nylon screen (200- $\mu$ m mesh) to obtain the total cell populations. After centrifugation, the cells were resuspended in serum-free RPMI 1640 medium and treated with  $\text{NH}_4\text{Cl}$ . Cell number and viability

were assessed microscopically using trypan blue exclusion.  $3 \times 10^6$  viable cells/ml of medium were incubated in culture medium supplemented with 10% (v/v) heat inactivated fetal bovine serum (FBS) and antibiotics (50  $\mu$ g/ml streptomycin and 50 units/ml penicillin), defined as basal medium, in culture plates. After incubation for 2 h at 37 °C in 95% air-5% CO<sub>2</sub>, the non-adhered cells were removed. The adhered MO monolayer showed 90% purity according to the morphologic analysis performed by Giemsa staining (Supplementary Figure 1, see section on supplementary data given at the end of this article) and nonspecific esterase staining. The MO from PCO rats (PCO MO), as well as the MO from control rats (control MO), were plated at a density of  $1 \times 10^6$  cells/well in a final volume of 1 ml in culture plates, preincubated in basal medium for 24 h and subsequently cultured for 24 h. The respective culture media were collected and used to stimulate ovaries and isolated ovarian cells from PCO rats (PCO ovaries), as well as from control rats (control ovaries).

#### ***Ovary incubation***

After sacrificing the rats, the ovaries from PCO and control rats were halved and preincubated in 1 ml of basal medium at 37 °C, in a 95% O<sub>2</sub>–5% CO<sub>2</sub> mixture. After 15 min, the incubation media were discarded and either 1 ml of basal medium or 1ml of MO secretions (PCO MO or control MO culture medium, respectively) was added (Forneris *et al.* 2008). Incubation was continued for 3 h, and then the medium was removed and stored at -20 °C until measurement of PGE<sub>2</sub> and nitrites release contents.

#### ***Obtaining and incubation of ovarian cells***

The procedures for harvesting and culturing the granulosa cells have been described previously (Erickson & Hsueh 1978, Aguado & Ojeda 1984). Briefly, granulosa cells from ovaries of PCO and control rats were harvested in RPMI 1640 medium, supplemented with 0.2% bovine serum albumin (BSA) fraction V, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) (10 mM) and ethylene glycol tetraacetic acid (EGTA) (6.8 mM), by needle-puncturing of the follicles. The

suspension was centrifuged at 100 x g for 10 min, and the cell pellet was washed in RPMI medium containing penicillin (50 U/ml) and streptomycin (50 µg/ml). After viability determination by trypan blue exclusion test, the cells were plated at  $3 \times 10^5$  viable cells/tube containing 1 ml of RPMI 1640 medium supplemented with androstenedione ( $10^{-7}$  M), L-glutamine (2 mM), penicillin (50U/ml), streptomycin (50 µg/ml) and ovine FSH (10 ng/ml). Androstenedione was included in all cultures and served as aromatase substrate (Erickson & Hsueh 1978). The cells were cultured in a humidified 95% v/v air and 5% v/v CO<sub>2</sub> incubator at 37 °C for 24 h. At the end of this period, the medium was discarded and replaced by fresh medium with antibiotics or macrophages secretions. The incubation was continued for 24 h and then the medium was stored at -20 °C until assayed for estradiol release. After harvesting granulosa cells by needle-puncturing of the follicles, the corpora lutea were removed, and the residual ovarian tissue, which is considered to be enriched in interstitial cells, was dissociated in 2 ml of RPMI medium containing 0.4% w/v collagenase type XI (Sigma-Aldrich Co.) and 0.1% BSA fraction V (Sigma-Aldrich Co.), by stirring at 37°C for 30 min in a metabolic shaking water bath and assisted by repeatedly drawing tissue fragments into a siliconized pipette. Five minutes before finishing the procedure, 0.001% of deoxyribonuclease type I (DNase I) (Sigma Inc, USA) was added. Subsequently, the digest was filtered through a nylon mesh and the suspension was centrifuged at 100 x g for 10 min. The cell pellet was washed twice in RPMI medium containing penicillin (50 U/ml) and streptomycin (50 µg/ml), centrifuged as described above, and finally resuspended in the same medium.  $5 \times 10^5$  cells were cultured in a humidified 95% v/v air and 5% v/v CO<sub>2</sub> incubator at 37 °C for 24 h. At the end of this period, the medium was removed, replaced by fresh medium with antibiotics or MO secretions, and the cells were cultured for an additional 24 h. Afterwards, the culture medium was stored at -20 °C until assayed for androstenedione release.

### ***Steroid assays***



Estradiol and androstenedione released in the media from the culture of granulosa and interstitial cells, respectively, were determined by RIA using specific antisera as described previously (Forneris & Aguado 2002). The assays sensitivities were less than 12 fmole/tube for estradiol and 0.02 ng/ml for androstenedione. For all steroids, the inter- and intra-assay coefficients of variation were less than 10%.

#### ***Nitrite assay***

Liquid incubations of ovaries from control and PCO rats were removed and analyzed for NO by assaying nitrites, which is a stable product of NO oxidation, using the Griess reagent; the absorbance was read at 540 nm (Egami & Taniguchi 1974). The intra-assay coefficients of variation were less than 10%. The results were expressed as micromoles of nitrite per milliliter ( $\mu\text{mol/ml}$ ).

#### ***Histological studies***

After incubation with basal medium or MO secretions, the ovaries were fixed in Bouin's solution. The samples were dehydrated in graded series of ethanol and embedded in paraffin. Sections of 5  $\mu\text{m}$  in thickness were obtained using a Microm HM 325 rotation microtome and stained with hematoxylin-eosin. Histological analysis was carried out using a computer-assisted image analysis system consisting of an Olympus BX-40 binocular microscope. The images were captured by a Sony SSC-DC50A camera and processed with Image Pro Plus 5.0 software under control of a Pentium IV computer.

#### ***Identification of apoptotic nuclei by TUNEL assay***

Apoptotic nuclei were identified in ovarian tissue using the DeadEnd <sup>TM</sup>Colorimetric TUNEL System (Promega, Madison, USA) according to the manufacturer's protocol. Briefly, nuclear DNA fragmentation was assessed in 5  $\mu\text{m}$  thick sections of ovarian tissue mounted on silane-coated slides, deparaffinized with xylene and then treated with a graded series of ethanol [100%, 95%, 85%, 70%, and 50% (v/v)] and rehydrated in PBS (pH 7.5). Tissues were then treated with proteinase K

solution for permeabilization and then refixed with 4% paraformaldehyde solution. Slides were then treated with recombinant terminal deoxynucleotidyl transferase (TdT) reaction mix and incubated in a humidified chamber at 37 °C for 1 h. The reaction was terminated by immersing the slides in 2 × SSC solution (saline-sodium citrate) for 15 min at room temperature. After blocking the endogenous peroxidases activity (by 0.3% hydrogen peroxide), slides were washed with PBS and then incubated with streptavidin horseradish peroxidase solution for 30 min at room temperature. Afterwards, the slides were incubated for 10 min with 3,3'-diaminobenzidine (DAB) as chromogen substrate. The sections were counterstained with hematoxylin, dehydrated, mounted and coverslipped. For negative control, incubation with TdT was omitted and the positive control was prepared by treating cells on a separate slide with DNase I. Cells showing dark brown staining from the colorimetric reaction were considered as positive for DNA fragmentation.

The results of the TUNEL assay were evaluated according to the signal intensity as negative (–) and positive (+). TUNEL positive staining was examined in 10 consecutive areas from 5 independent ovarian sections. A total of 50 fields, from control and treated groups, were analysed. The number of TUNEL-positive follicles with apoptotic cells was counted in independent ovarian cross sections from three different rats under microscope at x100 objective.

### ***Western blot analysis for iNOS***

Ovaries were homogenized in tris-HCL 50mM (pH 7.8) containing protease inhibitors. Protein was measured by the method of Lowry *et al.* (1951). 40 mg of proteins were mixed with 10 ml of sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 0.5 mM DTT, 0.02% bromophenol blue and 20% glycerol), boiled for 2–3 min and loaded into 8% SDS-PAGE gel. Protein molecular mass markers were always loaded on each gel. The separated proteins were transferred to PVDF membranes (Polyscreen NEF 1000 purchased from NEN Life Science Products) using a blot transfer system (BioRad Laboratories, Hercules, CA). After being blocked overnight with 5% BSA-TBS solution

(20 mM Tris, 500 mM NaCl, pH 7.5) at 48 °C, membranes were incubated with a primary rabbit anti iNOS polyclonal antibody solution (Santa Cruz Biotechnology) (1:1000 dilution), for 1 h at room temperature.  $\beta$ -actin expression was measured as a control for protein loading using a rabbit polyclonal antibody. After washing three times with TTBS (0.1% Tween 20, 100 mM Tris-HCl, pH 7.5, 150 mM NaCl), the membranes were incubated with an anti-rabbit IgG secondary antibody linked to peroxidase for 1 h at room temperature. The membranes were washed and the colour was developed using a Vectastain ABC detection system.

#### ***Prostaglandin E<sub>2</sub> radioimmunoassay***

PGE<sub>2</sub> was determined by radioimmunoassay (RIA) as it has been previously reported (Motta *et al.* 1999). PGE<sub>2</sub> was quantified in incubation medium from ovary, and in ovary homogenate from PCO and control rats. To extract PGE<sub>2</sub>, the samples were first acidified to pH 3.0 with 1 M HCl and then extracted three times with 1 ml of ethyl acetate. The ethyl acetate extracts were dried under an atmosphere of N<sub>2</sub> and stored at -20°C until prostaglandin RIA was performed. PGE<sub>2</sub> was quantified by using rabbit antiserum from Sigma Chemical Co (St. Louis, USA). The sensitivity was 10 pg/tube and the cross-reactivity was 100% PGE<sub>2</sub> and < 0.1% with other prostaglandins. Results are expressed as pg/mg of tissue or ml of medium.

#### ***RNA extraction and semiquantitative RT-PCR***

Total RNA was extracted from MO culture using TRIzol reagent. The semi-quantitative analysis of mRNA, was performed using a one-step RT-PCR method (Access RT-PCR system, Promega, Madison, USA). All components for RT and PCR were assembled in 50  $\mu$ l reactions containing 5x reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), 3 mM MgCl<sub>2</sub>, 10 mM dNTP mixture, 1  $\mu$ M of each gene specific primers, 2  $\mu$ g template RNA, 5 units of AMV reverse transcriptase and 5 units of Tfl DNA polymerase. The amplification of cDNA was performed under the following conditions: denaturation at 94 °C for 2 min followed by 35 cycles of denaturation at 94 °C for 30 s; annealing at

60 °C for Bcl2, NFkB and GAPDH, and at 61 °C for Bax, during 1 min; and extension at 72 °C for 2 min. The reaction was completed with a final extension at 72 °C for 7 min (thermal cycler). The following primers were used: Bax: (5'-GCGAATTGGAGATGAACTGG -3' sense, and 5'-GTGAGCGAGGCGGTGAGGAC-3' anti-sense), Bcl2 (5'-GCAACCGAACGCCCCGCTGTG -3' sense and 5'-GTGATGCAGGCCCCCACCAG-3' antisense), NFkB: (5'-AGCCCAGAAATATGGACCGT-3' sense and 5'-TCGAAATCTGAAGCCTCGCTGT-3' antisense), GAPDH: (5'-GGGCTGCCTTCTCTTGTGAC-3' sense and 5'-CGCCAGTAGACTCCACGACA-3' antisense). The predicted sizes of the PCR-amplified products were 366, 474, 180, and 325 base pairs (bp) for Bax, Bcl2, NFkB and GAPDH, respectively. The PCR products were resolved on 2% agarose gel electrophoresis, containing 0.5 mg/ml GelRed, and photographed with a Polaroid camera. Band intensities of RT-PCR products were quantified using NIH Image software. The relative abundance of each band was normalized according to the housekeeping GAPDH gene, calculated as the ratio of the intensity values of each product to that of GAPDH. Thus, results are expressed as mRNA/GAPDH in arbitrary units.

### ***Statistical analysis***

All data shown are expressed as mean  $\pm$  S.E.M. and analyzed using GraphPad Prism 5. Significant differences among means were considered at a level of  $P < 0.05$  and identified by one-way ANOVA and Tukey's test.

## **Results**

### ***Macrophage secretions action on the ovarian Bax, Bcl2 and NFkB mRNA expressions***

Presented in Fig. 1A are the results of the densitometric analysis of Bax mRNA (an apoptosis promoter) corrected for GAPDH expression (relative densitometric units), in PCO and control ovaries incubated with basal medium (basal conditions), control MO secretions and PCO MO

287 secretions. It is observed that in basal conditions, the Bax mRNA expression of the PCO ovary was  
288 not significantly different to that of the control ovary. Incubating control ovaries with either control  
289 or PCO MO secretions resulted in a similar Bax mRNA expression in relation to the control ovary in  
290 basal conditions. Furthermore, incubation of PCO ovary with control MO secretions did not show  
291 significant variations in the Bax mRNA expression with respect to PCO ovary in basal conditions.  
292 However, incubation of the PCO ovary with PCO MO secretions presented a significant increase of  
293 Bax mRNA expression with respect to PCO ovary incubated in basal conditions ( $P<0.01$ ), and  
294 therefore also showing a significant increase compared to the respective control ovary ( $P<0.001$ ).  
295 As shown in Fig 1B, no differences in the Bcl2 (an apoptosis inhibitor) mRNA expression were  
296 observed between PCO and control ovaries incubated with either basal medium, PCO  
297 MO secretions or control MO secretions. From the data shown in Fig. 1A and 1B, it can be  
298 concluded that incubation of PCO ovaries with PCO MO secretions, leads to an increase of the Bax  
299 to Bcl2 ratio with respect to PCO ovaries incubated in basal conditions ( $1.49 \pm 0.06$  vs  $1.16 \pm 0.03$ ;  
300  $P<0.05$ ), and also respect to the control ovaries incubated with PCO MO secretions ( $1.49 \pm 0.06$  vs  
301  $0.97 \pm 0.03$ ;  $P<0.05$ ).  
302 Incubation of PCO ovaries in basal medium shows no significant differences in the NFkB mRNA  
303 expression compared to the corresponding control ovaries. Furthermore, after incubating with both  
304 control and PCO MO secretions, no variations were observed in the NFkB mRNA expression of the  
305 control ovaries compared to the control ovary in basal conditions. However, PCO ovaries incubated  
306 with both control and PCO MO secretions, presented an increase of the NFkB gene expression  
307 compare to PCO ovaries incubated in basal conditions ( $P<0.05$  and  $P<0.01$ , respectively). Likewise,  
308 there was also an increase of the gene expression with respect to the corresponding control ovaries  
309 incubated with both control and PCO MO secretions, being this increase greater when PCO ovaries

were incubated with PCO MO secretions ( $P<0.05$  for control and  $P<0.01$  for PCO MO secretions) (Fig. 1C).

#### ***Macrophage secretions on the ovarian PGE<sub>2</sub> content and release***

Presented in Fig. 2A are the results of the PGE<sub>2</sub> content in the ovaries. It is observed that under basal conditions, the PGE<sub>2</sub> content was significantly increased in the PCO ovary respect to the control ovary ( $P<0.001$ ). It is also observed that incubating the control ovaries with both control and PCO MO secretions, does not show an appreciable variation of prostaglandin content with respect to ovaries incubated in basal conditions. However, incubation of the PCO ovary with both control and PCO MO secretions showed a significant decrease of PGE<sub>2</sub> content with respect to PCO ovary in basal conditions, and also compared to their respective control ovaries ( $P<0.001$ ), being this decrease more pronounced in the case of PCO ovary incubated with PCO MO secretions.

As shown in Fig. 2B, under basal conditions, the amount of PGE<sub>2</sub> released from the PCO ovary was significantly increased compared to the control ovary ( $P<0.001$ ). Incubating the control ovaries with both control and PCO MO secretions, resulted in no significant variation in the amount of PGE<sub>2</sub> released with respect to control ovaries incubated with basal medium. However, incubation of the PCO ovary with both control and PCO MO secretions showed a significant decrease of PGE<sub>2</sub> release with respect to PCO ovary in basal conditions ( $P<0.001$ ), being this decrease more pronounced in the case of PCO ovary incubated with PCO MO secretions. Therefore, incubating the PCO ovary with PCO MO secretions showed a decrease of PGE<sub>2</sub> release compared to the corresponding control ovary ( $P<0.05$ ).

#### ***Macrophage secretions on the ovarian nitrite release and iNOS protein expression***

Presented in Fig. 3A are the results of nitrite released from the ovaries. The release of nitrites from PCO ovary incubated in basal conditions showed an increase with respect to the nitrites released by the corresponding control ovary ( $P<0.01$ ). Stimulating control ovaries with both control and PCO

MO secretions, resulted in a similar increase of nitrite release compared to control ovaries in basal condition ( $P<0.001$ ). Furthermore, incubation of the PCO ovaries with control MO secretions showed an increase of nitrites released ( $P<0.01$ ) with respect to basal conditions, while no change was observed when incubating with PCO MO secretions. Thus, incubation PCO ovaries with PCO MO secretions showed a significant decreased of the nitrite released from the PCO ovaries in relation to the respective control ovary ( $P<0.001$ ).

Figure 3B shows the results of iNOS protein expression in the ovaries. Immunoblot analysis with an anti-iNOS monoclonal antibody demonstrated enhanced levels of iNOS protein expressions in PCO ovaries incubated in basal conditions relative to the corresponding control ovary ( $P<0.05$ ). Stimulation of the control ovaries with both control and PCO MO secretions, did not show changes of the iNOS expression with respect to that in basal conditions; while stimulating the PCO ovaries with both secretions resulted in a decrease of the iNOS expression relative to the PCO ovary in basal conditions ( $P<0.001$ ), being this decrease more important with PCO MO secretions. Therefore, incubating the PCO ovary with PCO MO secretions showed a decrease of iNOS protein compared to the corresponding control ovary ( $P<0.001$ ).

#### ***Effect of macrophage secretions on the steroids release from ovarian cells***

Incubating with MO secretions from PCO as well as from control rats had a stimulatory effect on the androstenedione release from interstitial cells of both control and PCO ovaries, compared to their respective basal values ( $P<0.001$ ). In all cases, including basal conditions, PCO ovaries released more androstenedione than control ovaries (Fig. 4A), being this difference greater when stimulating with PCO MO secretions. Figure 4B shows that after stimulation with both control and PCO MO secretions, estradiol release from control granulosa cells showed no significant differences compared to basal conditions. However, incubating the PCO granulosa cells with both secretions, resulted in a decrease of the estradiol release relative to the cells in basal conditions, and this

decrease was more pronounced when PCO granulosa cells were incubating with PCO MO secretions ( $P<0.05$  for control and  $P<0.01$  for PCO MO secretions).

#### ***Effect of macrophage secretions on the ovarian TUNEL staining***

In situ TUNEL analysis was used to detect the presence of apoptotic activity in ovarian sections (Fig. 5A-F). As expected, in basal conditions, the control ovary showed low or no staining, and absence of cystic follicles. Only a negligible amount of apoptosis in granulosa cells of secondary and antral follicles was observed (Fig. 5A). On the other hand, the PCO ovary showed TUNEL positive reaction in few primary follicles, an increased reactivity in secondary and antral follicles, as well as abundance of cystic follicles, compared to control (Fig. 5B). TUNEL staining was predominately localized in granulosa cells and in less extent on theca cells. It was observed that when incubating with PCO MO secretions, the TUNEL reactivity in secondary and antral follicles of control ovary increased (Fig. 5E), and even more in secondary and antral follicles of PCO ovary (Fig. 5F), compared with the respective ovary in basal conditions. TUNEL staining was seen in both granulosa as well as theca cells in PCO ovaries under stimulation with PCO MO secretions. The distribution of TUNEL positive reactions in granulosa cells in primary, secondary and antral follicles is illustrated in the Table 1.

#### **Discussion**

Cystic ovarian disease and/or polycystic ovarian syndrome (PCOS) are reproduction disorders that affect human beings and other species such as bovine, ovine, caprine and porcine (Garverick 1997, Jakimiuk *et al.* 2002, Silvia *et al.* 2002, Salvetti *et al.* 2004, Salvetti *et al.* 2007). The heterogeneity of the syndrome is reflected in many polycystic ovaries animal models (Mahajan 1988, Salvetti *et al.* 2004, Baravalle *et al.* 2006, Francou *et al.* 2008). Furthermore, estradiol valerate is one of the most widely used steroids to induce PCO in rats (Brawer *et al.* 1986, Rosa *et al.* 2003). On this



383 basis, in the current work, the effect of macrophage secretions on the polycystic ovarian apoptosis  
384 has been studied in order to gain further knowledge on the mechanisms determining PCO. The  
385 results show that macrophage secretions induce apoptosis in the PCO ovary.  
386 In the ovary, the mechanisms underlying decisions of life and death involve cross-dialogue between  
387 pro-apoptotic and pro-survival molecules through fetal and adult life, which are carried out by  
388 several molecular pathways, out of which the Bcl2 family, TNF $\alpha$  and caspases proteins appear to be  
389 important players. The Bax/Bcl2 ratio, which is thought to be a critical determinant of cell fate (a  
390 low ratio favours extended survival of cells, whereas a high ratio accelerates cell death) (Rueda *et*  
391 *al.* 1999, Tilly 2001, Skarzynski *et al.* 2005, Bas *et al.* 2011), was significantly higher in PCO than  
392 in control ovary incubated with PCO MO secretions, suggesting that these secretions may be  
393 involved in an imbalance among apoptotic and pro-apoptotic family members in the PCO ovary. In  
394 this sense, the NF $\kappa$ B mRNA expression was increased in the PCO ovary after incubation with the  
395 PCO MO secretions. It is known that the activation of this transcription factor, which induces the  
396 transcription of downstream target genes involved in the inflammatory and apoptotic process  
397 (Perkins 2007), is induced by TNF $\alpha$  in rat granulosa cells through phosphorylation of I $\kappa$ B  
398 (Gonzalez-Navarrete *et al.* 2007). Considering the pro-apoptotic role for TNF $\alpha$  in reproductive  
399 tissues (Kaipia *et al.* 1996, Hussein 2005, Haider & Knöfler 2009), and having observed that  
400 secretions from spleen MO of PCO rats contain more TNF $\alpha$  than secretions from control MO  
401 (Figueroa *et al.* 2012), the induction of NF $\kappa$ B mRNA expression in the PCO ovary after incubation  
402 with control MO secretions, which was even more accentuated with PCO MO secretions, could be  
403 associated, at least till some extend, to the TNF $\alpha$  levels of the MO secretions. In addition, after  
404 incubation with PCO MO secretions, an increase of the apoptotic nuclei number in the granulosa  
405 cells of PCO ovary was detected by TUNEL staining. All these findings suggest that PCO MO

secretions induce apoptosis in the PCO ovary. Furthermore, immune cells play an important role in the pathology of PCOS, as suggested by the high concentration of white cells in PCO ovary (Bukulmez & Arici 2000, Wu *et al.* 2004, 2007). Thus, resident macrophage population could possibly modulate the cell death in the PCO ovary.

In the ovary, resident macrophage and other white cells, as well as follicular granulosa and theca cells, are known to produce prostaglandins (Takaya *et al.* 1997). In particular, PGE<sub>2</sub> has been shown to mediate the ovarian follicle growth in the rat *in vivo* (El-Nefiawy *et al.* 2005). In the present work, it was found that the content of PGE<sub>2</sub> in the PCO ovary incubated with basal medium was higher than in control ovary, as it was also observed with the PGE<sub>2</sub> release. This agrees with results from other authors who found an increased PGE<sub>2</sub> release from dehydroepiandrosterone- or letrozole-induced polycystic ovaries in rats, and also from polycystic ovary granulosa cells collected from women (Navarra *et al.* 1996, Jabbour *et al.* 2009, Rezvanfar *et al.* 2011). Interestingly, both control and PCO MO secretions decreased the PGE<sub>2</sub> content of PCO ovary, and this decrease was more pronounced with PCO than with control MO secretions. It is known that NO, which is produced by the resident and infiltrating ovarian MO, and also by ovarian cells (Bukulmez & Arici 2000, Wu *et al.* 2004, 2007), stimulates cyclooxygenase-2 (COX-2) activity, which is the rate-limiting step in prostaglandin synthesis (Chandrasekharan & Simmons 2004). Therefore, the fact that iNOS protein expression and nitrite levels follow a similar pattern than the PGE<sub>2</sub> content, suggests that PGE<sub>2</sub> synthesis can be modified by PCO MO secretions.

The exact role of prostaglandins during apoptosis remains unclear. On the other hand, PGE<sub>2</sub> promotes survival of pathological cells by inhibiting apoptosis (Sheng *et al.* 1998, Rask *et al.* 2006), as well as death cells by a pro-apoptotic effects (Bowolaksono *et al.* 2008). We have observed an inverse relationship between PGE<sub>2</sub> content and apoptosis of the PCO ovary after stimulation with PCO MO secretions, where a lower ovarian PGE<sub>2</sub> content and release, and a higher apoptotic nuclei

430 numbers, were found compared with the effect induced by control MO secretions. It is also known  
431 that PGE<sub>2</sub> suppress cell death induced by TNF $\alpha$  in ovarian bovine luteal cells (Bowolaksono *et al.*  
432 2008), and Kunisch *et al.* (2009) have shown that PGE<sub>2</sub> can act as a negative feedback molecule in  
433 the signalling pathway linking TNF $\alpha$  to pro-destructive matrix metalloproteinase (MMP1)  
434 production in fibroblasts cells. In addition, a high ovarian matrix metalloproteinase-2 (MMP2) has  
435 been found to be increased in PCO rats compared with healthy controls (Rezvanfar *et al.*  
436 2014). Therefore, since from our previous results we know that the TNF $\alpha$  level of PCO MO  
437 secretions is higher than control MO secretions (Figuerola *et al.* 2012), it is conceivable that the low  
438 PGE<sub>2</sub> content of PCO ovary stimulated with those secretions can contribute to apoptosis promotion.  
439 Moreover, an apoptotic role of androgen has been recognized in reproductive tissues. In this sense,  
440 global knockout of the androgen receptor in female mice leads to reduced fertility, with reduced  
441 numbers of antral follicles and increased granulosa cells apoptosis (Shiina *et al.* 2006, Walters *et al.*  
442 2008, Tyndall *et al.* 2012). We have previously shown that PCO MO secretions decrease the  
443 conversion of androstenedione to estradiol in the PCO ovary, leading to an increase of  
444 androstenedione (Figuerola *et al.* 2012). This was also observed in the current study when PCO  
445 ovarian cells were incubated with PCO MO secretions, where an increase of androstenedione release  
446 from interstitial cells and a decrease of estradiol release from granulosa cells occurred. This effect  
447 can be attributed, at least in part, to the high TNF $\alpha$  content of PCO MO secretions. It has been  
448 reported that TNF $\alpha$  activates transcription of an inducible repressor form of 3',5'-cyclic adenosine 5'-  
449 monophosphate-responsive element binding modulator, and represses P450 aromatase in ovarian rat  
450 granulosa cells (Morales *et al.* 2006). In addition, we have previously observed an increase of IL6  
451 mRNA expression in PCO MO, compared with control MO, which was even greater after treatment  
452 with testosterone (Figuerola *et al.* 2012), indicating that IL6 mRNA abundance seems to be

modulated, as it was observed with  $\text{TNF}\alpha$ , by testosterone environment. Since an enhanced IL6 production may attenuate estradiol production, partially by inhibiting the expression of aromatase mRNA in rat granulosa cells (Tamura *et al.* 2000), it is possible that the increased IL6 expression of PCO MO may contribute to the steroidogenic ability of the MO secretions to decrease the androstenedione conversion to estradiol in the PCO ovary.

Moreover, the low  $\text{PGE}_2$  content and NO production found in the PCO ovaries stimulated with MO secretions could contribute to the androgen environment.  $\text{PGE}_2$  stimulates Cyp19 expression, the key gene of estrogen biosynthesis, in rat granulosa cells (Cai *et al.* 2007). In relation to NO, it is known that the in vitro synthesis of estradiol is inversely regulated by NO in ovarian rat cells (Dave *et al.* 1997) and also in human granulosa-luteal cells cultures, being the NO capable of directly inhibiting the activity of aromatase or indirectly decreasing the levels of the mRNA of the enzyme (Snyder *et al.* 1996).

Considering the present results and knowing that testosterone stimulates the  $\text{TNF}\alpha$  and IL6 production by MO, it is reasonable to suggest that the increase of androgens stimulated in ovarian cells by PCO MO secretions could in turn stimulate the cytokine production from MO, thus maintaining an apoptotic vicious cycle in the PCO ovary.

Despite the bidirectional relationship between pro-inflammatory cytokines and the androgen excess in PCOS is still unclear, our results suggest that the detrimental effect of up-regulation of proinflammatory cytokines of spleen macrophages, could contribute to explain the lack or loss of reproductive capacities observed in the PCOS. Also, the possibility that MO secretions released into the general circulation can act on hypothalamic-pituitary sites, and subsequently produce changes in the ovary, cannot be discard.

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667    **Declaration of interest**

668    The authors declare that there is no conflict of interest that could be perceived as prejudicing the  
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## Legends

### Figure 1A

Macrophage secretions on the ovarian Bax mRNA expression. Ovaries from control (C) and polycystic (PCO) rats were stimulated with either basal medium (basal) or culture medium of C and PCO macrophages (macrophage secretions) for three hours. Target mRNA was normalized by the level of GAPDH mRNA. Values are mean  $\pm$  S.E.M. of three independent experiments, each experiment performed with three C and three PCO rats. \*\*\* $P < 0.001$  compared with respective control.

### Figure 1B

Macrophage secretions on the ovarian Bcl2 mRNA expression. Ovaries from control (C) and polycystic (PCO) rats were stimulated with either basal medium (basal) or culture medium of C and PCO macrophages (macrophage secretions) for three hours. Target mRNA was normalized by the level of GAPDH mRNA. Values are mean  $\pm$  S.E.M. of three independent experiments, each experiment performed with three C and three PCO rats. \*\*  $P < 0.01$ ; \*  $P < 0.05$  compared with respective control.

### Figure 1C

Macrophage secretions on the ovarian NF $\kappa$ B mRNA expression. Ovaries from control (C) and polycystic (PCO) rats were stimulated with either basal medium (basal) or culture medium of C and PCO macrophages (macrophage secretions) for three hours. Target mRNA was normalized by the level of GAPDH mRNA. Values are mean  $\pm$  S.E.M. of three independent experiments, each experiment performed with three C and three PCO rats. \*\*  $P < 0.01$ ; \*  $P < 0.05$  compared with respective control.

### Figure 2A

Macrophage secretions on the ovarian prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) content. Ovaries from control (C) and polycystic (PCO) rats were stimulated with either basal medium (basal) or culture

medium of C and PCO macrophages (macrophage secretions) for three hours. Values are mean  $\pm$  S.E.M. of three independent experiments, each experiment performed with three C and three PCO rats. \*\*\* $P < 0.001$  compared with respective control.

### Figure 2B

Macrophage secretions on the ovarian prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) release. Ovaries from control (C) and polycystic (PCO) rats were stimulated with either basal medium (basal) or culture medium of C and PCO macrophages (macrophage secretions) for three hours. Values are mean  $\pm$  S.E.M. of three independent experiments, each experiment performed with three C and three PCO rats. \*\*\* $P < 0.001$ ; \* $P < 0.05$  compared with respective control.

### Figure 3A

Macrophage secretions on the ovarian nitrite release. Ovaries from control (C) and polycystic (PCO) rats were stimulated with either basal medium (basal) or culture medium of C and PCO macrophages (macrophage secretions) for three hours. Values are mean  $\pm$  S.E.M. of three independent experiments, each experiment performed with three C and three PCO rats. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$  compared with respective control.

### Figure 3B

Macrophage secretions on the ovarian iNOS protein expression. Ovaries from control (C) and polycystic (PCO) rats were stimulated with either basal medium (basal) or culture medium of C and PCO macrophages (macrophage secretions) for three hours. The corresponding graph shows integrated optical density of the bands. Values are mean  $\pm$  S.E.M. of three independent experiments, each experiment performed with three C and three PCO rats. \*\*\* $P < 0.001$ ; \* $P < 0.05$  compared with respective control.

### Figure 4A

Effect of macrophage secretions on the androstenedione release from ovarian cells. Interstitial cells (IC) from control (C) and polycystic (PCO) rats were stimulated with either basal

medium (basal) or culture medium of C and PCO macrophages (macrophage secretions) for 24 h. Values are mean  $\pm$  S.E.M. of three independent experiments, each experiment performed with three C and three PCO rats.  $**P<0.01$ ;  $*P<0.05$  compared with respective control.

#### **Figure 4B**

Effect of macrophage secretions on the estradiol release from ovarian cells. Granulosa cells (GC) from control (C) and polycystic (PCO) rats were stimulated with either basal medium (basal) or culture medium of C and PCO macrophages (macrophage secretions) for 24 h. Values are mean  $\pm$  S.E.M. of three independent experiments, each experiment performed with three C and three PCO rats.  $**P<0.01$ ;  $*P<0.05$  compared with respective control.

#### **Figure 5**

In-situ detection of apoptosis by TUNEL in ovarian sections of control and PCO rats. Representative photomicrographs of control (A, C and D) and estradiol valerate exposed (B, E, and F) rat ovary sections are shown. Ovaries from control and PCO rats were incubated for 6h in basal medium (Basal) or macrophage secretions from control (MO Control) and PCO (MO PCO) rats. Nuclear dark brown staining shows a positive reaction. Arrowheads indicate isolated apoptotic cells and arrows indicate apoptotic follicles. In basal conditions, increased apoptosis on PCO ovary is evident as compared to negligible apoptosis in control ovary (figure A-B). PCO MO secretions increase TUNEL reactivity in control ovary and even more in PCO ovary (figure E-F). Control MO secretions do not modify TUNEL reactivity in control or PCO ovary compared to respective basal (figure C-D). TUNEL staining was predominately localized in granulosa cells. Magnification X100.

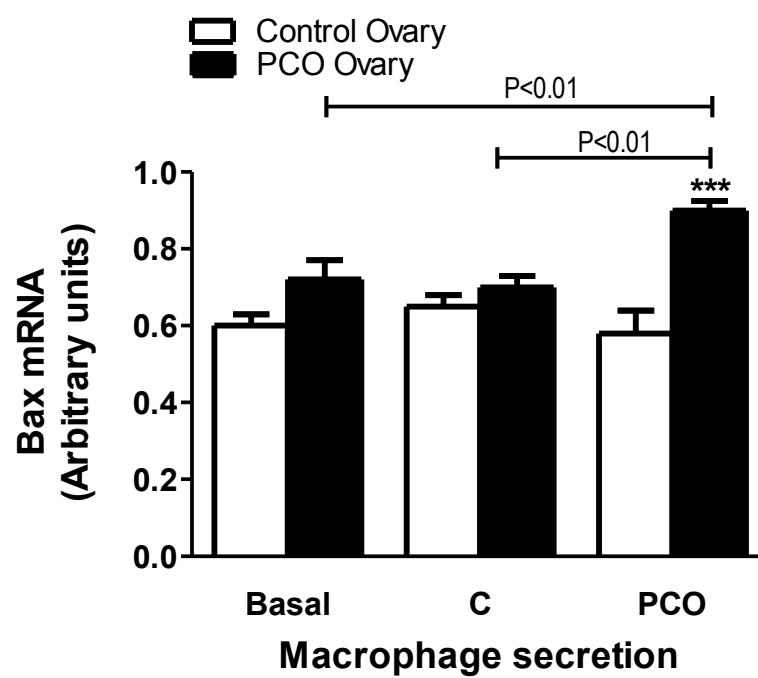
#### **Table 1**

*In situ* detection of apoptosis by TUNEL in ovarian sections from control and PCO rats treated, or untreated, with macrophages secretions.

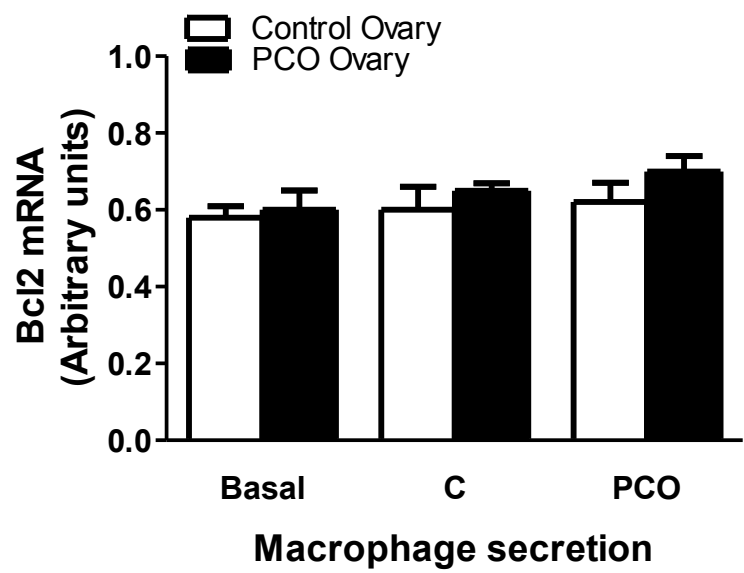
Adult cycling Holtzman control and PCO rats (injected with 2 mg of estradiol valerate) were sacrificed at estrus day. Ovaries from control and PCO rats were incubated for 6 h in basal medium (Basal) and macrophage secretions from PCO rats (MO PCO). Then, the ovaries were collected and processed for detection of apoptosis by TUNEL. Intensity of the positive TUNEL reaction: (-) negative; (+/-) weak positive; (+) positive; (++) strong positive; (+++) strong positive including theca and stromal cells; Ab, absence of respective type of follicle.

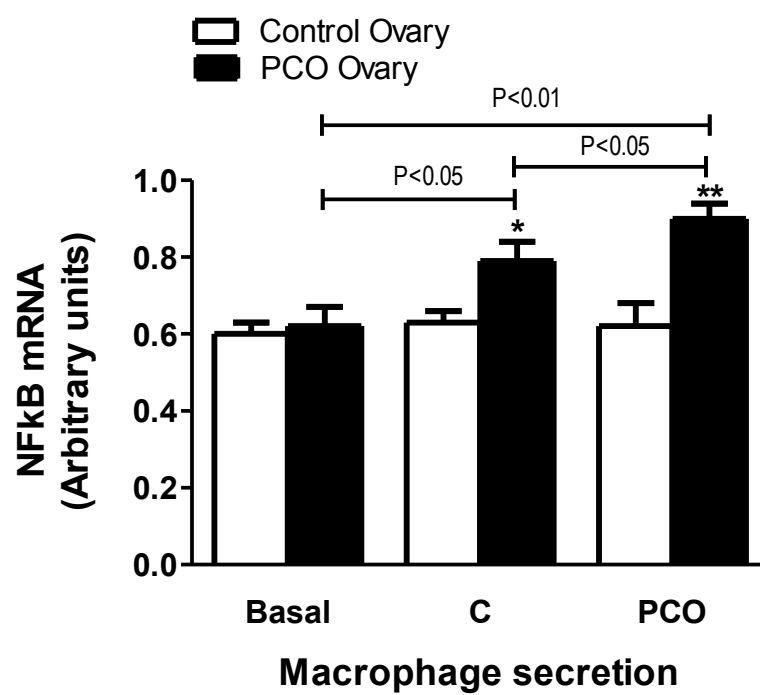
Legend to Supplementary Figure 1:

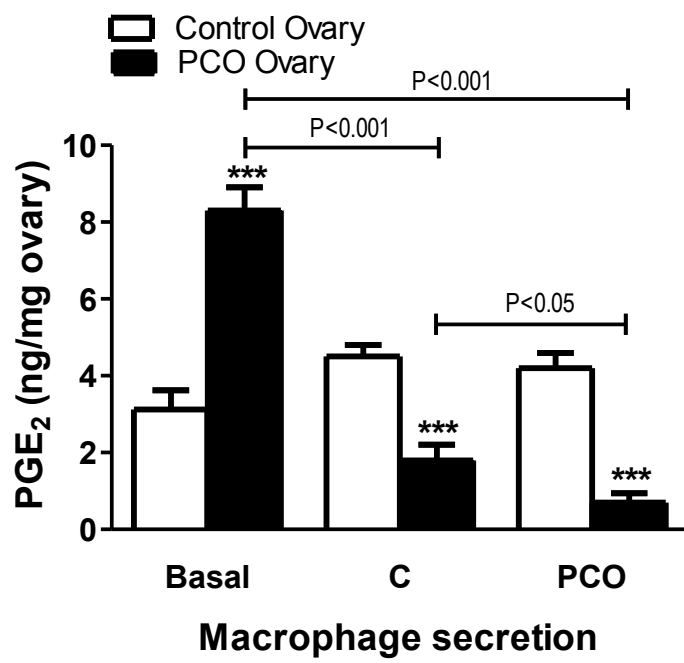
Giemsa staining of the adhered macrophage cells, after incubation of the splenic cells in RPMI media for 2 h, at 37 °C in 95% air-5% CO<sub>2</sub>. The morphology was determined by light microscopy, and the percentage of macrophage cells was obtained on 500 total cells counted in different microscopic fields taken at random. Magnification x400.

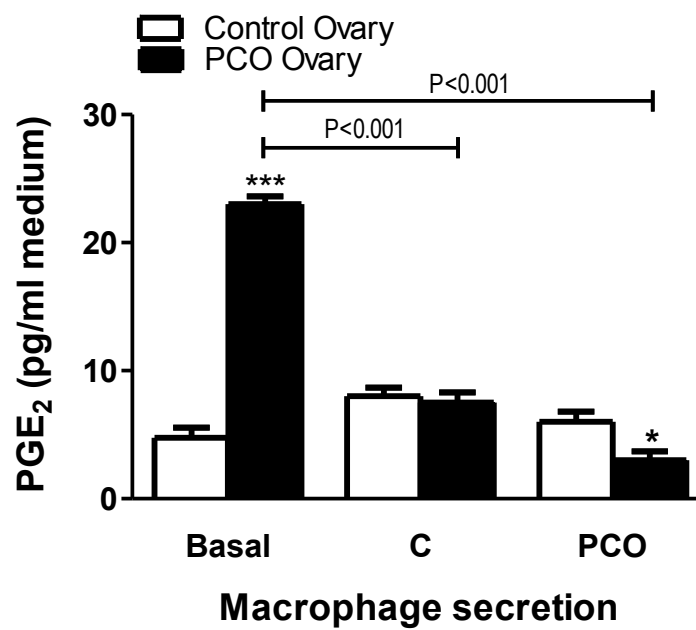


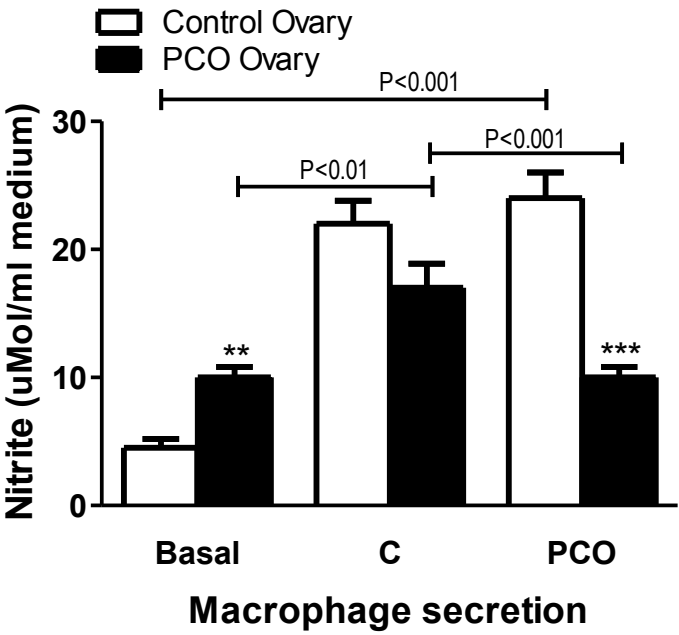


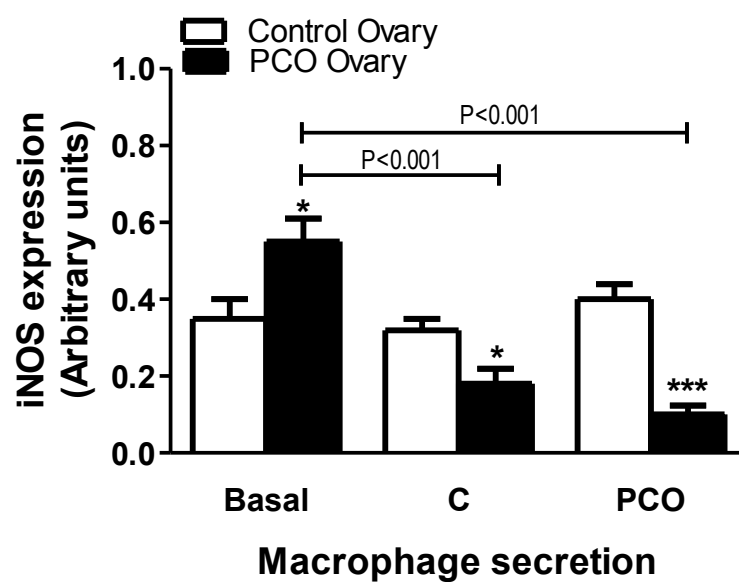


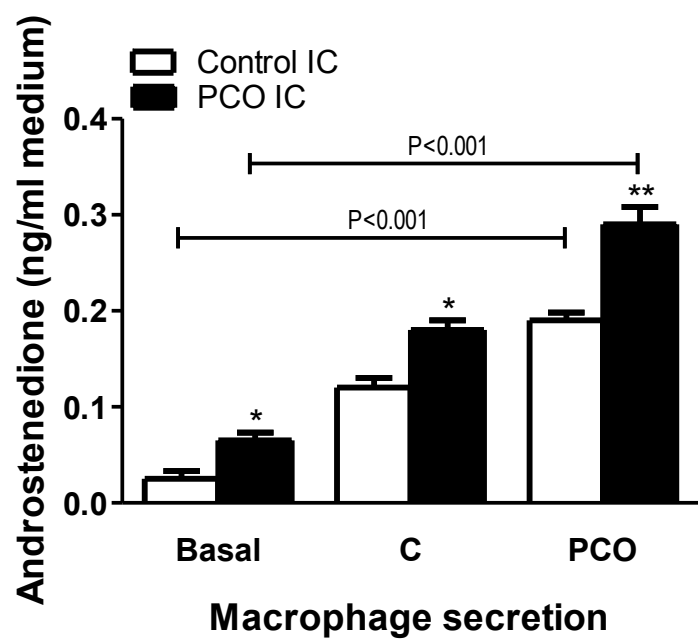


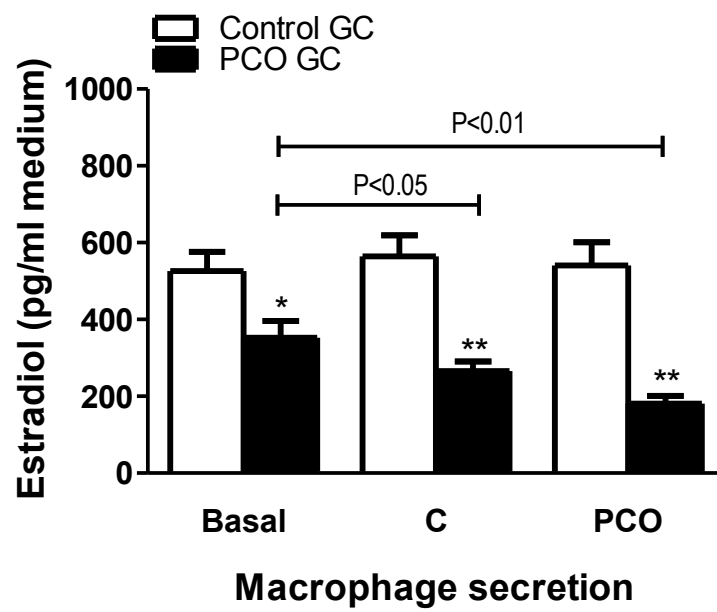














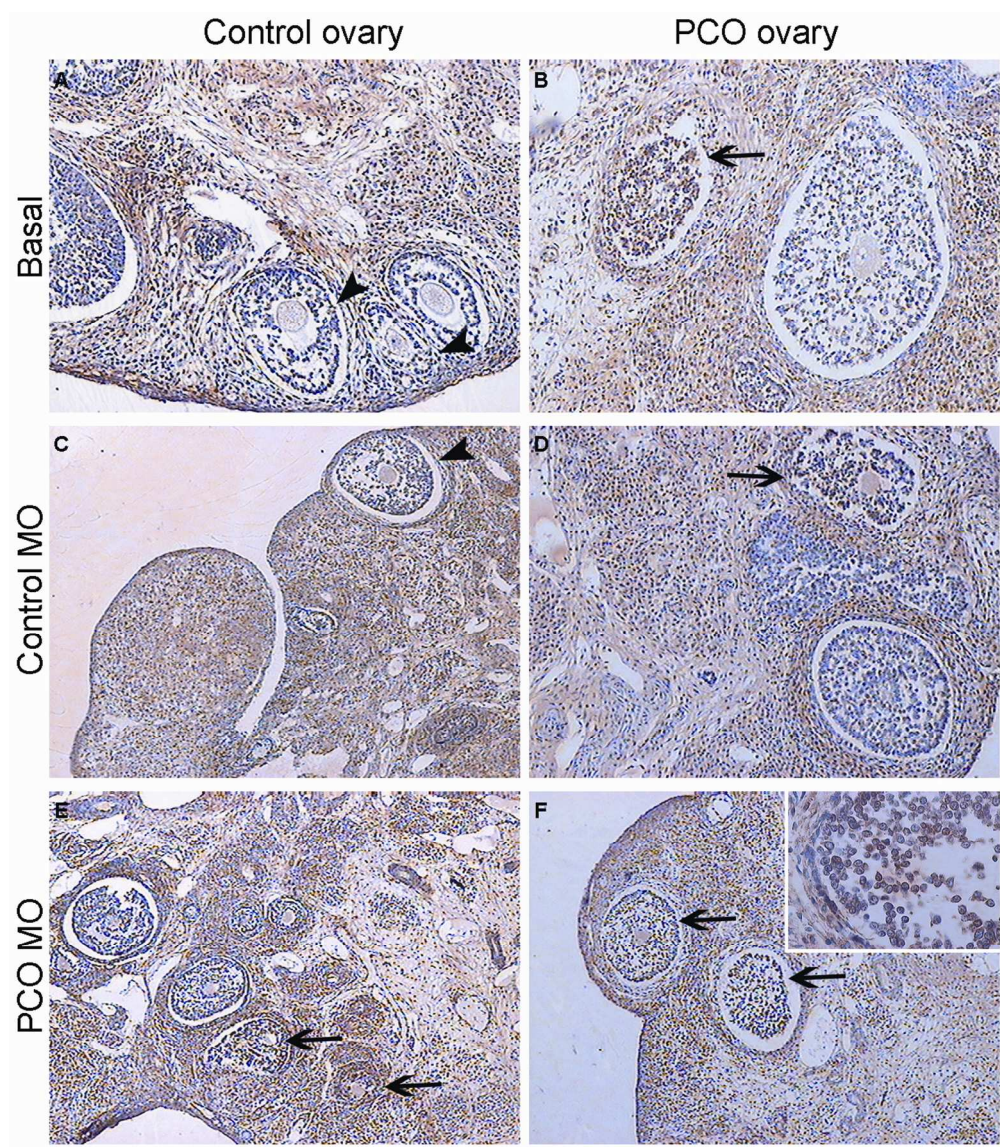


Figure 5

In-situ detection of apoptosis by TUNEL in ovarian sections of control and PCO rats. Representative photomicrographs of control (A, C and D) and estradiol valerate exposed (B, E, and F) rat ovary sections are shown. Ovaries from control and PCO rats were incubated for 6h in basal medium (Basal) or macrophage secretions from control (MO Control) and PCO (MO PCO) rats. Nuclear dark brown staining shows a positive reaction. Arrowheads indicate isolated apoptotic cells and arrows indicate apoptotic follicles. In basal conditions, increased apoptosis on PCO ovary is evident as compared to negligible apoptosis in control ovary (figure A-B). PCO MO secretions increase TUNEL reactivity in control ovary and even more in PCO ovary (figure E-F). Control MO secretions do not modify TUNEL reactivity in control or PCO ovary compared to respective basal (figure C-D). TUNEL staining was predominately localized in granulosa cells. Magnification X100.

115x131mm (300 x 300 DPI)

**Table 1.** *In situ* detection of apoptosis by TUNEL in ovarian sections from control and PCO rats treated, or untreated, with macrophages secretions.

GROUP	Follicles			
	Primary	Secondary	Antral	Cystic
Control (Basal)	-	-	+/-	Ab
PCO (Basal)	+	++	++	++
Control + MO PCO	-	+	++	Ab
PCO + MO PCO	++	+++	+++	+++